Journal of
Applied Bioanalysis

openaccess
http://dx.doi.org/10.17145/jab.20.004

ORIGINAL RESEARCH

Investigation of Potential in vivo Cleavage of Biotherapeutic Protein by Immunocapture-LC/MS

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Citation:
Chen L, Shan J, Roos D, Yu H. Investigation of Potential in vivo Cleavage of Biotherapeutic Protein by Immunocapture-LC/MS.
J Appl Bioanal 6(1), 12-25 (2020).

Editor:
Dr. Morse Faria, PPD Laboratories, Richmond, VA 23230, USA.

Received: September 06, 2019.
Revised: October 31, 2019.
Accepted: November 10, 2019.

Funding & Manuscript writing assistance:
The authors have declared that no funding nor writing assistance was utilized in the production of this article.

Financial & Competing interests:
All authors are employees of Boehringer Ingelheim Pharmaceuticals Inc. USA. The authors have declared that no competing interest exist.

OBJECTIVES: In recent years, there has been increasing demand to understand in vivo biotransformation of biotherapeutics and its impact on pharmacokinetics, efficacy, immunogenicity and safety. Liquid chromatography/mass spectrometry has been the primary technology for such investigation. In this study, a biotherapeutic protein was investigated for potential linker cleavage or truncation in vivo following intravenous administration to monkeys.

METHODS: Plasma samples were collected at a series of time points post dose on different days, prepared via immunocapture purification and enrichment, and analyzed by LC/MS. Two methods, intact/subunit LC/high-resolution mass spectrometry analysis and multiple reaction monitoring peptide analysis, were applied.

RESULTS: The biotherapeutic protein was found to remain intact in vivo and no linker cleavage or truncation was observed.

CONCLUSIONS: The combination of the two methods represents a generic approach and can be applied to the investigation of in vivo cleavage for other biotherapeutics.

KEYWORDS: Intact protein analysis, high resolution MS, multiple reaction monitoring, immunocapture-LC/MS, in vivo cleavage, biotherapeutics.

INTRODUCTION

In the last 30 years, biotherapeutics have become an important part of modern medicine and many have been developed with a diverse range of specific targets to treat various illnesses including cancer, heart disease, multiple sclerosis, anemia, and rheumatoid arthritis, etc. Biotherapeutics are produced by living organisms such as mammalian cells, viruses and bacteria and the end-product is determined by a wide range of factors including manufacturing process [1,2]. Critical quality attributes (CQA) can potentially impact safety and/or efficacy of the product, which includes product-related variant heterogeneity such as size (aggregates, fragments), charge (deamidation, isomerization), glycosylation (afucosylation, galactosylation, high mannose, sialylation), oxidation (methionine, tryptophan), sequence variants,

c-terminal clipping, glycation, disulfide linkage, etc. [3-5]. Physical, chemical, biological or microbiological properties or characteristics of biologics should be within an appropriate limit, range, or distribution to ensure the desired product quality [6]. Mass spectrometry (MS) plays an indispensable role in CQA assessment, particularly for investigating post-translational modifications. MS analysis allows specific investigation on the N- and C-termini of each chain (for the presence and relative levels of pyroglutamate at the N-terminus and lysine truncation at the C-terminus of the heavy chain), free sulfhydryl and disulfide bridge structure, glycosylation, and other post-translational modifications [7-9].
In recent years, there has been increasing demand to understand in vivo biotransformation of biotherapeutics and its impact on pharmacokinetics (PK), efficacy, immunogenicity and safety after administration [10-12]. In vivo biotransformation of proteins has been known to take place by reactions such as oxidation, deamidation and isomerization of amino acids and truncation of peptide chains, release of the small-molecule drugs (payloads) from antibody-drug-conjugates (ADCs), and hydrolysis of the PEG-protein bonds in PEGylated products [13, 14]. Trastuzumab, a mAb for breast cancer treatment, has been found to undergo extensive deamidation at the binding region in patients with potential implications on its activity [15]. In vivo biotransformation of a novel fusion protein tetranectin/apolipoprotein A1 (TN-ApoA1) have been investigated at the intact protein level and two catabolites were identified in rabbit by LC/high-resolution MS (HRMS) [16]. Biotransformation can also be followed by monitoring multiple surrogate peptides from different parts of the molecule. For instance, two peptides of dulaglutide (a fusion protein of GLP-1 linked to the Fc domain of IgG4), an N-terminal peptide from the GLP-1 part and the other from the Fc domain, were quantitated to assess molecular linker cleavages or truncation [17]. While the concentrations of the Fc peptide remained high after dosing, the concentrations of the GLP-1 peptide decreased and correlated with the concentrations found for the intact protein. The results suggested biotransformation, and probably proteolytical cleavage, in the N-terminal part of the drug. A similar approach was used to study the in vivo fate of a protein–drug conjugate [18]. In a concerted approach, a combination of ligand binding assay, mAb subunit analysis by LC/MS and peptide mapping by LC/MS were applied to investigate mAb drug concentration in circulation, biotransformation, and biotherapeutic drug product quality in vivo [19]. Structural modifications such glycation [20, 21], high mannose [22] and Fc deamidation [23] have been found to have effect on the clearance, efficacy or safety of the mAbs in vivo. Immunoaffinity capture coupled with a capillary electrophoresis–mass spectrometry system was used to characterize intact protein mass analysis of a wild type Fc-FGF21 construct and a sequence re-engineered Fc-FGF21 construct from an in vivo study [24]. A number of truncated forms were observed and the time courses of the various proteolytic products were identified and compared between the two constructs. The abundances of the intact and truncated forms were used to provide the basis to semi-quantify PK properties of the two protein forms. These case studies demonstrated the importance of monitoring CQAs and possible biotransformation from in-life sample sets as a control for product quality, and perhaps in certain instances safety or efficacy [25, 26].
Here we report a study to assess potential linker cleavage or truncation of an IgG1 fusion protein in vivo following administration to cynomolgus monkeys, using combination of immuno-purification with intact/subunit LC/HRMS analysis as well as multiple reaction monitoring (MRM) peptide analysis. In vivo modifications to the biotherapeutic protein can be readily detected by this approach. It can be applied as a generic approach for in vivo CQA assessment, particular for new modalities of biotherapeutics.

MATERIALS AND METHODS

The biotherapeutic protein XYZ was provided by Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT, USA) and produced in-house (Figure 1). The protein (MW ~201,400 Da) contains a mAb (human IgG1) backbone with C-terminus linked to two identical sin-

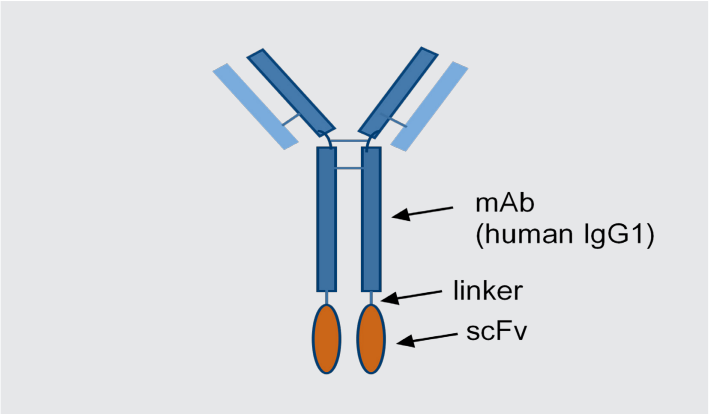

Figure 1. Structure of biotherapeutic protein XYZ. The protein (MW ~ 201,400 Da) contains a mAb (human IgG1) backbone with C-terminus linked to two identical single-chain variable fragments (scFv) from another mAb. The linkers consist of several amino acids.

gle-chain variable fragment (scFv) from another mAb. The linkers consist of several amino acids. A truncated version containing the human IgG1 backbone only (MW ~149,368 Da) was provided in house. Isotope labeled peptide internal standards (IS), GWYFDLWGS-Lys[$^{13}C_6$,$^{15}N_2$] and GPSVFPLAPSSLys[$^{13}C_6$,$^{15}N_2$], were synthesized at Biopeptek (Malvern, PA, USA). Trypsin treated with N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and EZ-Link Sulfo-NHS-LC-biotinylation kits were obtained from Thermo Scientific (Rockford, IL, USA). Streptavidin Magnesphere Particles and Peptide:N-glycosidase F (PNGase F) were purchased from Promega (Madison, WI, USA). RapiGest was purchased from Waters (Milford, MA, USA). Goat anti-human IgG H&L (monkey preadsorbed) was obtained from Novus Biologics (Centennial, CO, USA). Cynomolgus monkey blank plasma was purchased from Bioreclamation (Westbury, NY, USA). All other lab chemicals, reagents and buffer solutions were obtained from Sigma Aldrich (St. Louis, MO, USA), Thermo Scientific or Invitrogen (Grand Island, NY, USA).

Monkey study samples

Protein XYZ was given intravenous (IV) to cynomolgus monkeys (1 male and 1 female per dose group) at 0, 1, 10, 30 and 100 mg/kg weekly for three weeks. For intact/subunit analysis, plasma samples collected at 24 hr following the last (third) dose on Day 15 were used. For the MRM analysis, plasma samples collected at different time points following the first dose (Day 1) and the last doses (Day 15) from the 1, 10, 30 and 100 mg/kg dose groups were used. Prior to the immunocapture process, high drug concentration plasma samples were diluted with blank monkey plasma to approximately 50 µg/mL for intact/subunit analysis and to approximately 25 µg/mL for MRM analysis.

Plasma samples

Protein XYZ and its truncated version hIgG were supplied in 10 mg/mL buffer solutions. Plasma standards at various concentrations were prepared by spiking the buffer solutions to blank monkey plasma. Peptide international standards (IS) were made to a final concentration of 250 ng/mL in water. The standards were store at -80°C prior to use.

Biotinylation

Goat anti-human IgG H&L (monkey preadsorbed) was biotinylated using EZ-Link Sulfo-NHS-LC-biotinylation kits following the vendor instructions. Typical biotin incorpora-

tion was determined to be on an average of 3 biotins per IgG molecule as measured by HABA/Avidin assay [27].

Immunocapture

Streptavidin magnetic beads were freshly prepared. Approximately 200 µg of magnetic beads were used per µg of drug in the monkey plasma. Total amounts of beads to be used per batch were dependent on batch size. Beads were mixed thoroughly before use. An aliquot of beads at 1 mg/mL were transferred to a 50 mL vial, and washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBS-T). The beads were resuspended in TBS-T buffer to yield a 5 mg/mL concentration. Immunocapture was performed on a Kingfisher Flex automated system (Thermo Scientific, Rockford, IL, USA). For each sample, a 300 µL aliquot of washed beads was mixed with 75-150 µL of biotinylated anti-human IgG antibody and 500 µL of TBS-T buffer in a 96 deep-well plate. The mixture was incubated at room temperature for 1 hour on the Kingfisher Flex, washed twice with 450 µL of TBS-T, and resuspended in 300 µL of TBS-T. Then 150 µL of monkey plasma was added and incubated for another 2 hrs at room temperature with gentle mixing. The beads were separated, washed three times with 300 µL of TBS-T and once with 300 µL of water, and eluted in 150 µL of 25 mM HCl. The eluent was neutralized to pH 8 by adding 15 µL of 1 M Tris-HCl (pH 8).

Intact mass LC/MS analysis

LC/MS intact mass analysis was conducted on a Sciex 6600 TripleTOF system (Sciex, Framingham, MA, USA) coupled with an Acquity UPLC system (Waters, Milford, MA, USA). A 30 µL aliquot of immunocapture-processed sample was injected to the LC/MS system for analysis. LC separation was carried out on a Waters BEH C4 column (50 x 4.6 mm, 2 µm) at 80 °C. The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The LC gradient program started at 5% B, held for 2 min, then to 90% B over 4 min, followed by a wash period of 2.5 min at 90% B at 0.4 mL/min. The MS was run in positive ionization mode and the key parameters were as follows: GS1 40, GS2 40, CUR 40, TEM 350, ESI voltage +5500, TOF scan range from 900 Da to 4000 Da with 80 bins to sum.

Subunit LC/MS analysis

A 100 µL aliquot of immunocapture-processed sample was treated with 5 µL of 100 mM tris(2-carboxyethyl)phosphine (TCEP) for 1 hr to reduce disulfide bonds. The reduced sample was divided into two aliquots. One aliquot was directly injected to LC/MS for heavy chain and light chain analysis. The other aliquot was deglycosylated using PNGase F to remove the glycans from the heavy chain. PNGase F was diluted 4-fold with 100 mM ammonium bicarbonate and 2 µL of the diluted solution was added to the sample. The mixture was incubated at 37°C for 2 hr followed by TripleTOF LC/MS analysis. The same HPLC column and MS parameters used for intact mass analysis were applied. The LC gradient program started at 10% B for 1 min, and then to 50% B over 15 min at a flow rate of 0.4 mL/min. The column was washed with 90% B for 2 min followed by equilibration at 10% B for 3 min.

MRM analysis

To each immunocapture-processed sample, 5 µL of 0.1% RapiGest surfactant in 100 mM ammonium bicarbonate and 5 µL of 100 mM TCEP in 100 mM ammonium bicarbonate were added. The samples were incubated at 60°C for 1 hr. Five µL of stable-isotope labelled peptide internal standard solution and 5 µL of 200 mM iodoacetamide in 100 mM ammonium bicarbonate were added. The samples were incubated in the dark at room temperature for 30 min. After incubation, 5 µL of 100 mM $CaCl_2$ and 5 µL of 6 µg/µL trypsin were added. The samples were incubated overnight at 37°C with gentle mixing. On the

next day, the digestion was quenched with 5 µL of 20% trifluoroacetic acid, followed by incubation at 37°C for 45 min. The samples were centrifuged at 3800 rpm for 10 minutes. Ten µL of the processed sample was injected onto a Waters BEH C18 reversed phase column (2.1 x 50 mm, 1.7 µm) on a Waters Acquity UPLC coupled to a Sciex Triple Quad 6500 mass spectrometer. The mobile phase A consisted of 0.1% formic acid in water and B was 0.1% formic acid in acetonitrile. The LC gradient program was as follows: 5% B held for 0.8 min and then to 35% over 2.5 min at 0.3 mL/min. The column was held at 45°C. Typical LC/MS/MS operating parameters were as follows: GS1 50, GS2 50, CUR 30, TEM 500, and ESI voltage +5500. The MRM parent to product ion transitions were as follow: GPSVFPLAPSSK: 593.827→699.404, GWYFDLWGK: 586.282→ 928.456, GPSVFPLAPSS[Lys$^{13}C_6$,$^{15}N_2$]: 597.827→707.404, and GWYFDLWG[Lys$^{13}C_6$,$^{15}N_2$]: 590.282→ 936.456.

Data analysis

The intact and subunit LC/MS data was processed using PeakView 2.2.0 and Bio Tool Kit 2.2.0 from Sciex. The input spectrum isotope resolution was set to low (5000). An input m/z rang of 1900 to 3100, 1100 to 2300 and 800 to 3700 was used for intact, subunit light chain and subunit heavy chain deconvolution, respectively. For the MRM analysis, Microsoft Excel was used to calculate the MS response ratios of the Fc extension scFv domain peptide over the IgG CH1 peptide. The peptide responses were normalized with respective IS.

RESULTS AND DISCUSSION

A biomatrix such as plasma presents considerable challenges for applying LC/MS to in vivo CQA assessments. Plasma is very complex matrix that contains thousands of proteins and protein isoforms in a wide concentration range [28]. When no target protein purification is employed, LC/MS sensitivity is significantly compromised due to matrix interference [29]. For high-sensitivity LC/MS applications, immunocapture is the most effective way to remove matrix and improve assay sensitivity and robustness [30, 31].

In this work, an immunocapture method using an anti-human IgG Ab, was developed and implemented. The anti-human IgG Ab was monkey preadsorbed and therefore would not cross-react with endogenous monkey plasma proteins. Upon the addition of the anti-human IgG to the monkey plasma, only the drug as well as its potential IgG backbone containing fragments were bound to the biotinylated Ab to form drug-Ab complexes, which were then immobilized on the streptavidin magnetic beads. After separated from plasma, the beads were washed and eluted under acidic conditions. The sample was then subjected to intact/subunit analysis (top-down) or trypsin digestion followed by MRM analysis (bottom-up). The overall workflow is illustrated in **Figure 2**.

Multiple analytical approaches can be utilized to characterize biotherapeutic molecules in their intact state. Intact mass LC/MS analyzes the whole protein molecule and determines the accurate protein mass, and therefore is a direct approach for assessing protein intact mass. Biotherapeutic drugs can be further analyzed at their subunit level following reduction to separate light and heavy chains, deglycosylation to remove glycans and/or cleavage at the hinge region of IgG via immunoglobulin-degrading enzyme of *Streptococcus pyogenes* (IdeS) digestion. The subunit analysis facilitates exact molecular weight determination of their heavy and light chains. With intact/subunit analysis, in vivo modifications on the protein molecule such as truncation, linker cleavage, glycation, etc., can be readily detected by comparing the molecular weights measured in the study samples with a reference standard. In contrast, the MRM approach breaks down the protein into peptides via enzymatic digestion such as trypsin, and then analyzes specific peptides by LC-MS/MS. The MS response ratio of peptides from different regions of the protein along the amino acid sequence can be used to assess potential molecule cleavage. As peptides produce higher MS responses than proteins due to their smaller sizes, the

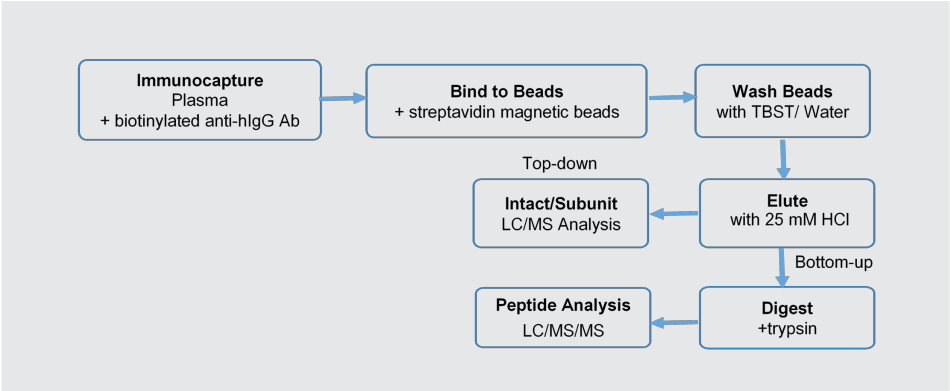

Figure 2. Immunocapture-LC/MS workflow. The anti-hFc antibody for immunocapture was pre-biotinylated prior to adding into monkey plasma. After immunocapture, streptavidin magnetic beads were added, separated from plasma and washed, then the drug was eluted from the beads and subjected to either intact/subunit mass analysis (top-down) or trypsin digestion followed by LC/MS/MS analysis (bottom-up).

MRM approach is usually more sensitive than intact/subunit analysis [32, 33]. For a comprehensive molecule cleavage assessment, we applied both the intact/subunit and MRM approaches and considered the two complementary to each other.

Intact/subunit analysis

A 50 µg/mL of protein XYZ monkey plasma standard containing 0%, 2%, 5% and 10% the truncated IgG fragment via the C-terminus linker cleavage were analysed in order to establish the detection limit for the intact analysis. Both protein XYZ and its linker cleavage truncated fragment were detected and identified based on their masses and glycosylation patterns. The measured molecular weight (based on the G0F/G0F glycoform) was 201,411–201,413 Da for the drug and 149,373–149,375 Da for the fragment via the loss of both scFv domains, which agreed with theoretical molecular weight 201,404 and 149,368, respectively (**Table 1**). The tolerance for mass error of intact analysis was 10 Da. The truncated fragment was detected and identified by accurate mass matching at 2–10 % levels. Thus, the limit detection of the intact analysis was determined to be 2% truncated products in 50 µg/mL protein XYZ in the monkey plasma. Likewise, the limit detection for protein XYZ was determined to be 2 µg/mL based on the intact analysis of a series of plasma standards at concentrations ranging from 1 to 50 µg/mL.

For the *in vivo* intact analysis, the monkey plasma collected at 24 hrs after the last dose on Day 15 of the 1, 10, 30 and 100 mg/kg dose groups (1 male and 1 female per dose group) were used. Along with the study samples, plasma standards at 2 and 50 µg/mL

Table 1. Comparison of masses of intact, subunit and linker cleavage fragments obtained from LC/MS analysis of study samples and spiked plasma standards with theoretical masses.

Analysis	Study samples	Plasma Standards	Theoretical mass
Intact mass analysis	201,410 - 201,413	201,411 - 201,413	201,404
TCEP reduction subunit analysis light chain	23,348 - 23,350	23,348 - 23,350	23,353
TCEP reduction subunit analysis heavy chain	77,363 - 77,364	77,363 - 77,366	77,369
TCEP/PNGase F subunit analysis heavy chain	75,918 - 75,923	75,920 - 75,921	75,923
Linker cleavage mAb fragment	Not detected	149,373 - 149,375 (- 2 scFv)	175,386 (- 1 scFV) 149,368 (- 2 scFv)

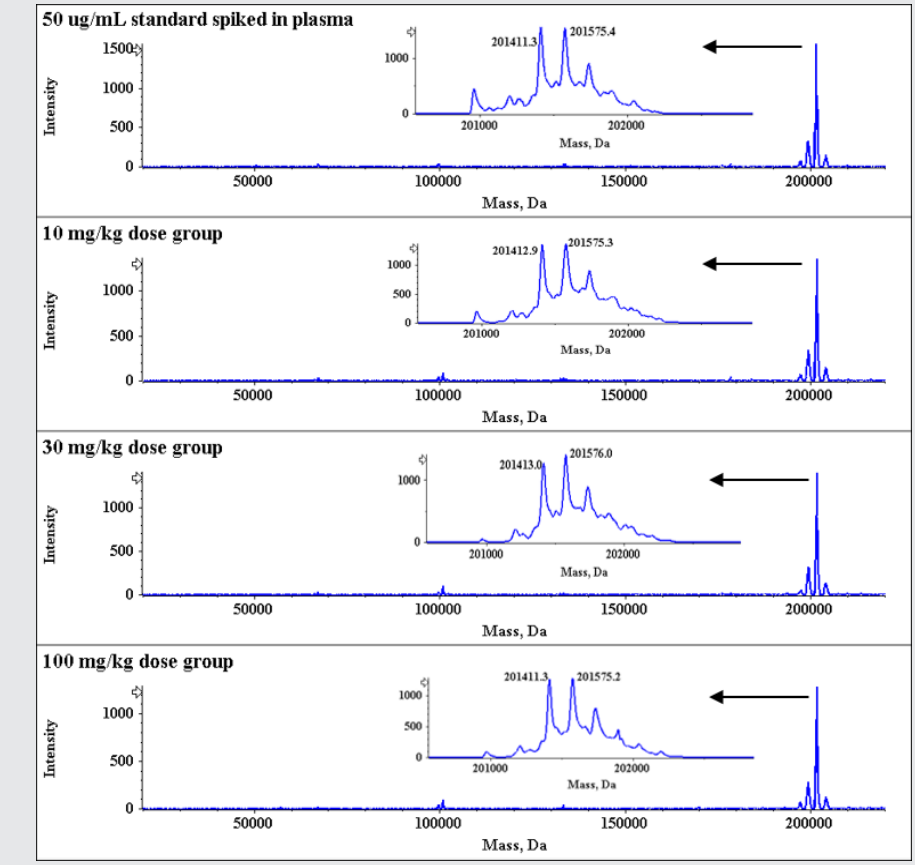

Figure 3. Protein XYZ intact mas analysis. Representative deconvoluted mass spectra from a 50 µg/mL standard spiked in blank monkey plasma, and 24 hr post-dose plasma samples from male monkeys of the 10, 30 and 100 mg/kg dose groups following the last doses on Day 15. Inserts are expanded views of intact mass molecular peaks with glycosylation patterns.

drug were assayed and served as references. The drug concentrations in the monkey study samples (determined by ELISA) varied significantly among dose groups, ≤ 0.269 µg/mL in the 1 mg/kg dose group monkeys whereas 18.0 – 577 µg/mL in the 10, 30 and 100 mg/kg dose groups. Samples with drug concentration higher than 50 µg/mL were diluted to approximately 50 µg/mL which was the immunocapture capacity. Representative deconvoluted LC/MS spectra of the 50 µg/mL monkey plasma standard and the male monkey plasma samples from the 10, 30 and 100 mg/kg dose groups are shown in **Figure 3**. As the drug concentrations in the 1 mg/kg dose group monkeys were below the intact analysis detection limit of 2 µg/mL, no deconvoluted MS was obtained. Protein XYZ was clearly detected in the 10, 30 and 100 mg/kg dose group samples. Molecular weight of the drug determined from the study samples was 201,410 - 201,413 Da, which was in agreement with the theoretical mass (201,404 Da) as well as the measured mass from the plasma standards (**Table 1**). Furthermore, the same glycosylation pattern was observed in the study samples and standards, as shown in **Figure 3**. The two C-terminus extension scFv were identical, and the expected masses for the linker cleavage products were 175,386 (loss of one scFv) and 149,368 (loss of both scFv). In all the monkey study samples, only the intact molecular peak was detected and no linker cleavage or any

truncation fragments were observed. In some samples, a small peak at half of the intact mass was found but it was likely an artifact due to false deconvolution which usually yielded half or one-third of the correct mass [34]. The results thus indicated that protein XYZ remained intact and no linker cleavage or truncation fragments were observed in the Day 15 – 24 hr post-dose study samples. Subunit analysis was also performed. The immunocapture-processed samples were reduced using TCEP to light and heavy chains and then injected to LC/MS for analysis. Representative deconvoluted LC/MS spectra of the monkey plasma standards and study samples after TCEP reduction are shown in **Figure 4**. The theoretical molecular weight of light chain and heavy chain with scFv was 23,353 Da for the light chain and 77,369 Da for the heavy chain, respectively (**Table 1**). The measured mass from the 50 µg/mL plasma standard was 23,348–23,350 Da for the light chain and 77,363–77,366 Da for the heavy chain. The measured mass in the monkey study samples was 23,348–23,350 Da for the light chain and 77,363–77,367 Da for the heavy chain, which were consistent with the theoretical masses as well as the masses obtained from the 50 µg/mL plasma standard. Neither truncation nor degradation peaks from either light or heavy chain were observed. In addition, the same heavy chain glycosylation pattern was observed between the study samples and the 50 µg/mL of plasma standard.

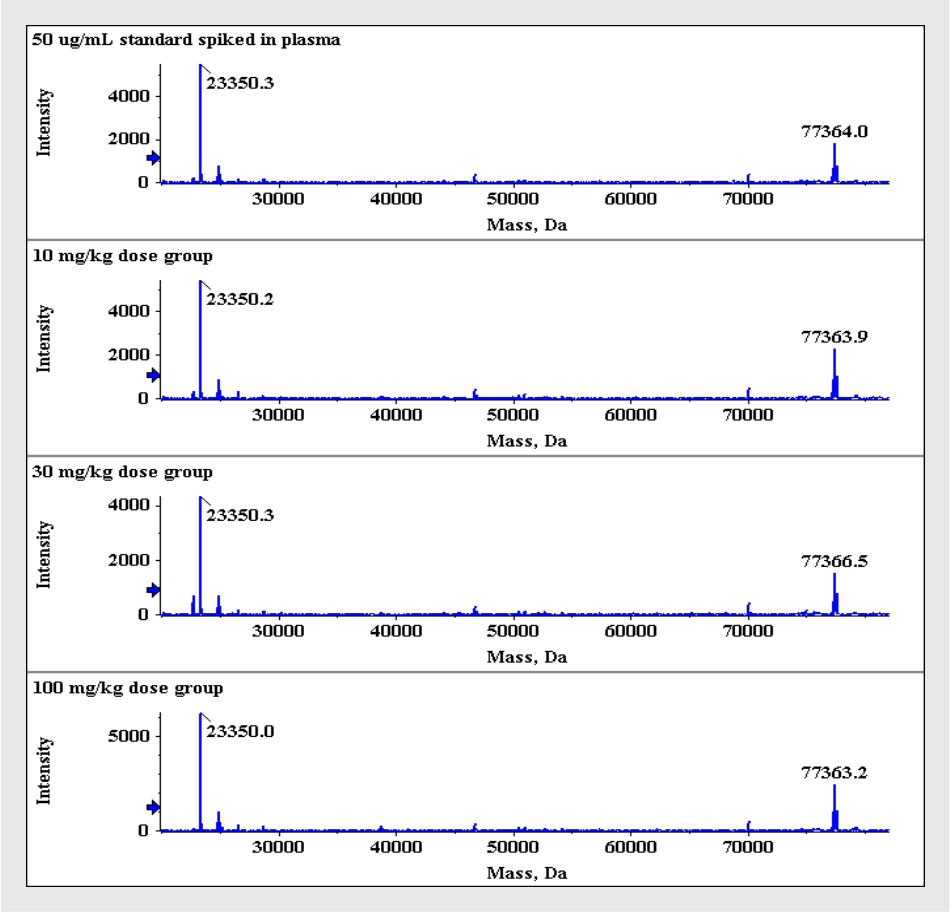

Figure 4. Subunit analysis of protein XYZ. Representative deconvoluted mass spectra after TCEP reduction from a 50 µg/mL standard spiked in blank monkey plasma, and 24 hr post-dose plasma samples from male monkeys of the 10, 30 and 100 mg/kg dose groups following the last doses on Day 15. The 23,350 Da and 77,363-77,367 Da peaks correspond to the light chain and heavy chain with a scFv, respectively.

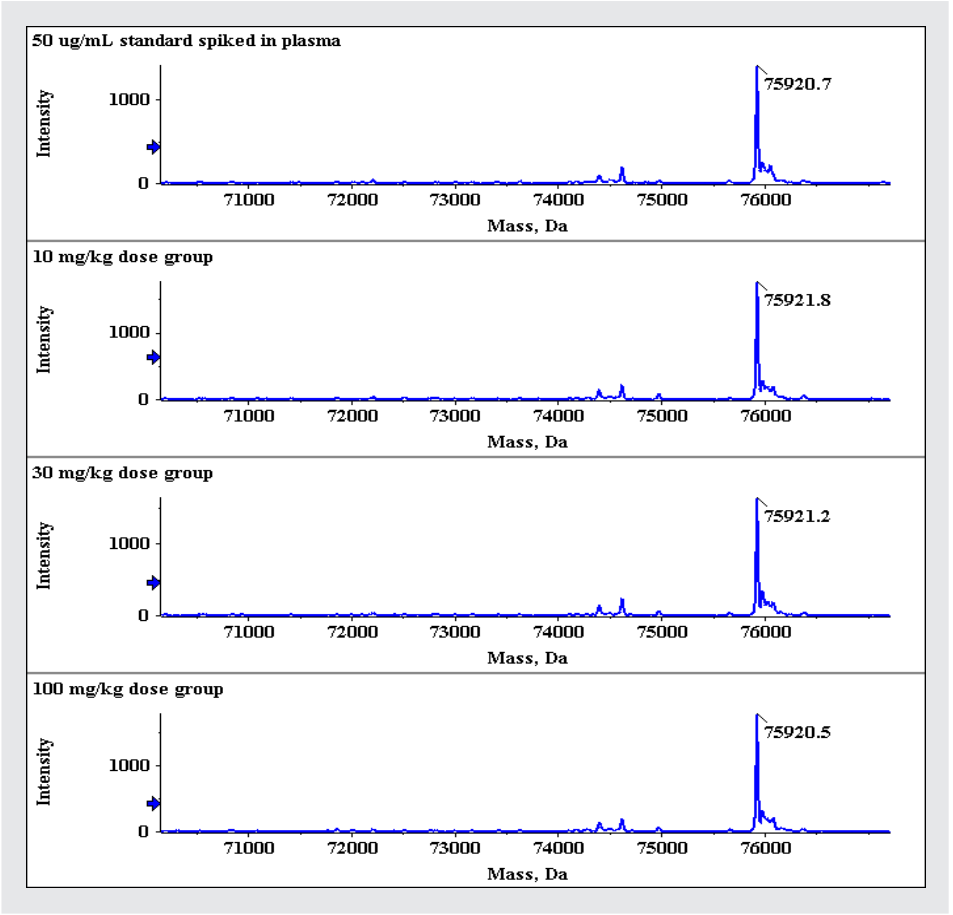

Figure 5. Heavy chain subunit analysis of protein XYZ after deglycosylation. Representative deconvoluted mass spectra of the heavy chain subunit after TCEP reduction and PNGase F deglycosylation from a 50 µg/mL standard spiked in blank monkey plasma, and 24 hr post-dose plasma samples from male monkeys of the 10, 30 and 100 mg/kg dose groups following the last doses on Day 15. The 75,920–95,922 Da peaks correspond to the heavy chain with a scFv minus glycan.

After TCEP reduction, the heavy chain was further deglycosylated using PNGase F and the molecular weight of the deglycosylated heavy chain was then determined. Representative deconvoluted LC/MS spectra of the 50 µg/mL monkey plasma standard and study samples after TCEP reduction and PNGase F deglycosylation are shown in **Figure 5**. The measured mass of the deglycosylated heavy chain from the study samples was 75,918 –75,923 Da, which was consistent with the theoretical mass of 75,923 Da as well as the measured mass (75,920–75921 Da) from the plasma standards (**Table 1**). Therefore, the deglycosylated subunit analysis further confirmed that protein XYZ remained intact in the monkey study samples.

MRM analysis

Possible linker cleavage was also assessed by monitoring MS response ratios of the tryptic peptides from the scFv domains to the CH1 domain. A scFv domain peptide GWYFDLWGK and an IgG CH1 domain peptide GPSVFPLAPSSK were monitored. These two peptides were unique to the drug and were not found in blank monkey plasma digest.

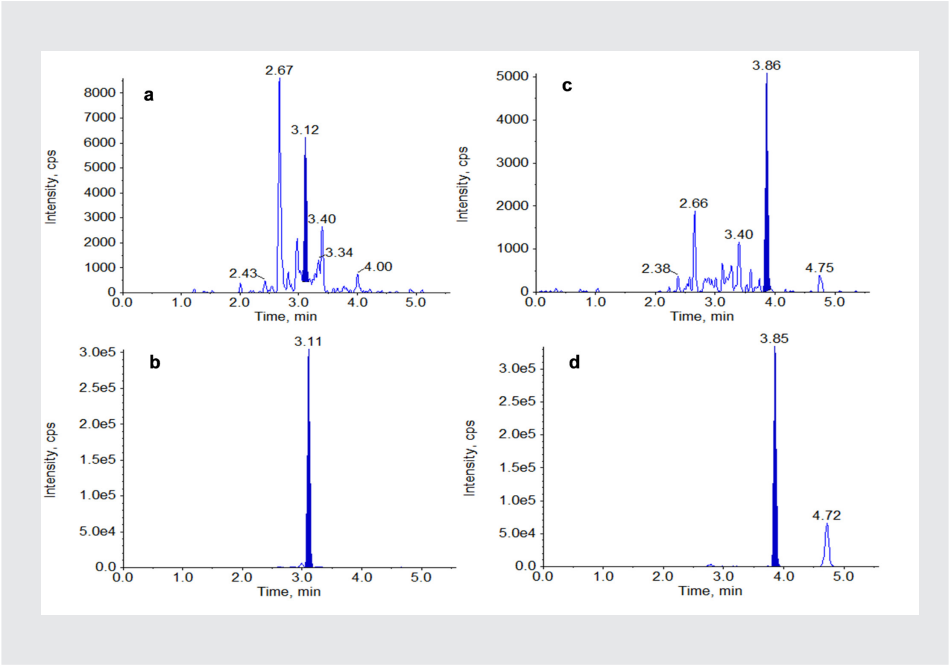

Figure 6. MRM analysis of a 50 ng/mL protein XYZ standard spiked in monkey blank plasma. Shown here are LC/MS/MS (MRM) chromatograms of the IgG CH1 domain peptide GPSVFPLAPSSK (a) and its internal standard GPSVFPLAPSS[Lys$^{13}C_6$, $^{15}N_2$] (b), and the C-terminus extension scFv domain peptide GWYFDLWGK (c) and its internal standard GWYFDLWG[Lys$^{13}C_6$, $^{15}N_2$] (d). Peaks correspond to the peptides are shown as integrated.

Calibration standards were prepared by spiking blank monkey plasma with the drug at a series of concentrations and used to assess the assay performance for each peptide. The MRM assay linear range was 50–25,000 ng/mL for both peptides. Back-calculated calibration standard concentrations were within ±15% from corresponding nominal values, indicating a good precision. The assay precision (% coefficients of variation) was ≤15.0% from three runs (data not shown). The assay provided a sufficient sensitivity, accuracy and precision for the MRM analysis of in vivo samples. Monkey samples with drug concentrations higher than 25,000 ng/mL were diluted to the linear range for MRM analysis.

Peptide MS signals depend on many confounding factors such as peptide ionization efficiencies, charge state distribution, LC gradient, trypsin digestion, etc. [35]. The intrinsic ionization properties [36] of a peptide vary depending on the constituent amino acids and their order. Differences and non-uniformity of the dynamic mobile phase constituents along the LC gradient program under which each peptide is introduced to MS ion source could potentially lead to variation in competition for ionization. Missed or nonspecific tryptic cleavages may introduce further variance. Therefore, the MS signal intensities arising from the two different peptide ions following electrospray ionization were not directly proportional to the amount (moles) of starting drug materials; the peptides derived from the same parent protein, even when present in stoichiometric amounts, would not generate the same number of ions and thus their attendant MS signal would vary. However, the ratios of the MS responses of the peptides from the same protein should remain constant in the monkey samples at various time points over the study duration. A constant ratio would imply no linker cleavage of the drug molecules for the duration of the study. On the other hand, linker cleavage would produce the human IgG1 fragment and the scFv domain

protein in the case of protein XYZ. Human IgG1 usually has a half-life of ~2 weeks [37] attributed to association with the neonatal Fc receptor [38]. In contrast, the small size of a scFv domain protein causes a short *in vivo* half-life owing to the rapid blood clearance and poor retention in the target tissues [39, 40]. As such, the peptide ratio would decreased over time, and a trend of decrease in the ratio over the study duration would thus be an indicator of in vivo linker cleavage.

Figure 6 shows the LC/MS/MS (MRM) chromatograms of the IgG CH1 domain peptide GPSVFPLAPSSK and the scFv domain peptide GWYFDLWGK and their respective internal standards from a 50 ng/mL protein XYZ standard spiked in monkey blank plasma. Despite some minor background peaks at the retention of the two peptides (especially for peptide GPSVFPLAPSSK; **Figure 6a**), both peptide peaks in the 50 ng/mL standard were accurately integrated for quantitation, demonstrating good assay specificity and sensitivity.

Monkey plasma samples collected at different time points following the first dose (Day 1) and the last doses (Day 15) from the 1, 10, 30 and 100 mg/kg dose groups were used for the MRM analysis. The monkey study samples were analysed along with a set of standards containing protein XYZ at series concentrations from 50 to 25,000 ng/mL. The LC/MS response ratio of the scFv domain peptide over the IgG CH1 domain peptide were determined over the course of the sampling time period for each animal in the monkey study. The average peptide ratios from all 8 animals were calculated for each time point on Day 1 and Day 15 and plotted over time in **Figure 7**. The coefficient of variation (CV%) for the averages was 8.2–18.2%. Overall, the CV% was within 20%, indicating a good assay precision. The ratios from the first post-dose time point, Day 1-0.083 hr, were used as the reference for comparison with all other time points on both Day 1 and Day 15.

A peptide ratio within 85–115% (or ±15%) of the first time point Day 1-0.083 hr post-dose, as indicated by the dotted lines in **Figure 7**, was within the assay variability and thus considered as an indication of no linker cleavage. The average response ratio of the scFv domain peptide GWYFDLWGK over the IgG CH1 domain peptide GPSVFPLAPSSK ranged from 1.10 to 1.25 at all the time points from Day 1 to the last sampling time point on Day 15 (**Figure 7**), which were within ±15% of the ratio (1.17) of the reference time

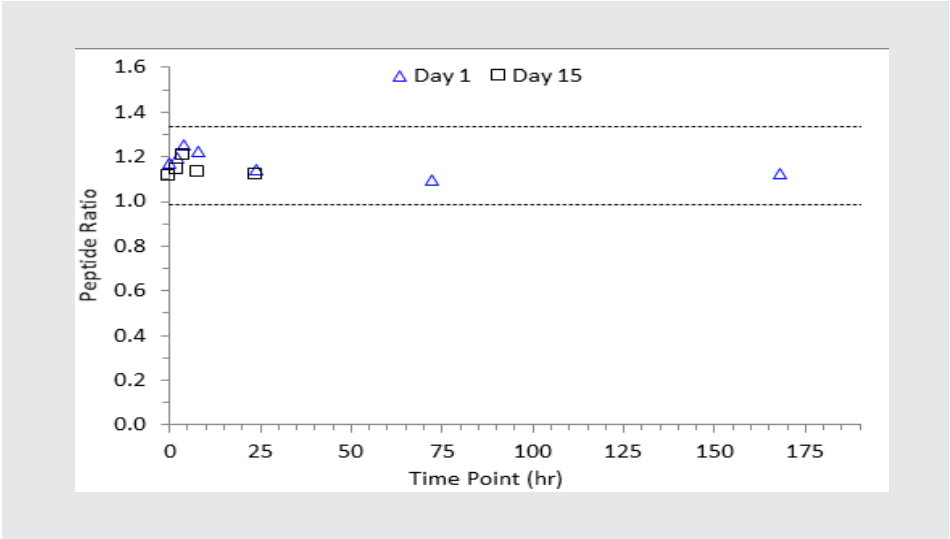

Figure 7. Peptide ratios of monkey study samples. The y-axis represents the average LC/MS response ratio of the scFv domain peptide GWYFDLWGK over the IgG CH1 domain peptide GPSVFPLAPSSK. The x-axis represents the time points post-dose following the first dose (Day 1) and last dose (Day 15). The dotted line indicates +/- 15% deviation from the value of the first data point on Day 1 – 0.083 hr.

point Day 1-0.083 hr. Thus, the MRM analysis indicated no linker cleavage of protein XYZ in the study samples.

CONCLUSION

In this study, both intact/subunit analysis and MRM analysis were applied to investigate potential in vivo cleavage of a biotherapeutic protein XYZ during a two-week study in monkeys. The intact/subunit LC/MS analysis was at the whole molecule level and provided a direct assessment on truncation and linker cleavage. On the other hand, the MRM analysis was more sensitive and provided a complementary assessment by monitoring the ratios of a specific scFv domain tryptic peptide to an IgG backbone peptide. Both approaches clearly demonstrated that the therapeutic protein XYZ remained intact and neither truncation nor linker cleavage was observed in vivo. It should be noted that sample purification and enrichment via immunocapture was a must for either approach. The combination of immunocapture with intact/subunit and MRM analysis represents a generic approach and can be applied to other biotherapeutic proteins.

Acknowledgment

The authors would like to thank Elsy Philip, Shirin Pagels, Ming Cheng, Adam Vigil, Christine Grimaldi for help during the course of this study.

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